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Original Article

Molecular Study of Vascular Endothelial Growth Factor Gene in Iranian Patients after Myocardial Infarction

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Abstract

Background: Stimulation of collateral artery growth (arteriogenesis) and/or capillary network growth (angiogenesis) would be beneficial to the patients with myocardial infarction. To understand the central role of vascular endothelial growth factor (VEGF) in biological angiogenesis, we performed molecular analysis of the VEGF gene in patients afflicted with acute myocardial infarction (AMI).

Method: Forty patients with AMI were divided into two groups according to the presence or absence of created collateral blood vessels in ischemic myocardial region. In these patients we also evaluated the possible relationship of plasma levels of VEGF and its growing ability of new blood vessels. The molecular *characterisation* of VEGF gene may highlight the presence of natural genomic variants which could facilitate the formation of new vessels in the ischemic area.

Results: The genomic analysis of VEGF gene did not reveal any mutations in the coding region, but showed the presence of four and one single nucleotide substitutions in the intronic region and 5'UTR respectively. The C to T nucleotide transition at position –7 of 5'UTR is located in a potential binding site for *Sp-1* transcription factor, which could probably affect the VEGF gene transcription.

Conclusion: The molecular study of VEGF gene showed that its coding region is highly conserved. Therefore, variations of angiogenesis could be due to the regulatory elements participating in this mechanism.
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Keywords ● Myocardial infarction ● VEGF ● gene ● serum level

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Introduction

Currently, the treatment of occlusive arterial disease includes medical therapy or revascularization techniques such as percutaneous transluminal angioplasty or bypass surgery.[1] However, this kind of therapy is not feasible for a large number of patients. The stimulation of collateral artery growth (arteriogenesis) and /or capillary network growth (angiogenesis) would be beneficial to these patients. In fact, various potent angiogenic growth factors have been tested for the treatment of peripheral arterial disease and ischemic heart disease.[2] In recent years, the efficacy of therapeutic angiogenesis

using vascular endothelial growth factor (VEGF) has been reported in patients with critical limb ischemia,[3] myocardial infarction,[4,5] and inoperable coronary artery diseases.[6] In order to understand the central role of VEGF on angiogenesis, we have performed a molecular analysis of VEGF gene in patients affected by acute myocardial infarction (MI) with and without formation of collateral blood vessels in the ischemic myocardial area.

We hypothesized that the molecular characterisation of VEGF gene in these patients might help to elucidate the presence of natural genomic variants that facilitating the formation of new vessels in ischemic area. Furthermore, in these patients the level of plasma VEGF was measured to elucidate if there is a relationship between its plasma concentration and the degree of development of new collateral blood vessels in the ischemic areas.

Patients and Methods

Patients

The present study was performed on 40 patients (25 male and 15 female) upon first presentation of acute MI referred to Nemazee Hospital, Shiraz University of Medical Sciences, Shiraz, Iran. Acute myocardial infarction was defined as resting chest pain lasting 30 minutes accompanied with elevating ST-segment evolving into pathological Q waves. It was confirmed by the presence of total creatinine kinase or MB fraction levels two times more than the upper normal limit. Angiography was performed using Seldinger method (Siemens model, Germany). A normal coronary artery was evidenced by the absence of any narrowing in coronary diameter. A narrowing of <70% (<50% in the case of the left main coronary artery) was considered non-significant coronary artery stenosis, and a narrowing of >70% (>50% in the case of the left main coronary artery) was regarded as significant coronary artery stenosis. All patients gave their written informed consent.

Polymerase chain reaction

Blood samples (EDTA) were collected and dispatched in dry ice to the Hemophilia and Thrombosis Center in Milan-Italy.

Genomic DNA was purified from peripheral blood leukocytes by salting-out method.[7] Five pairs of primers were designed to amplify all eight exons plus intron/exon junctions and 300 bp of 5' untranslated region (UTR) of the VEGF gene. The polymerase chain reaction (PCR) was performed with a 50 µl containing one unit of AmpliTaq®DNA polymerase (*Applied* Biosystem), 200 µM of each primer and 200 ng of genomic DNA. Primer sequence and annealing temperature of primers are summarized in Table 1.

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PCR products were purified by *QIA quick PCR purification Kit (QIAGEN,* Hilden, Germany) and directly sequenced using an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, Foster City, CA; USA) with the same primers used for the amplification reactions.

VEGF plasma levels

To determine the VEGF plasma levels, 8 ml of venous blood were collected using EDTA as anticoagulant and immediately centrifuged at 4,000 g for 10 minutes. Aliquots of plasma were prepared and stored at -80°C. VEGF plasma levels were measured using a commercially available enzyme immunoassay (Quantikine, Hum Ana, VEGF Immunoassay, R&D Systems, Oxon; UK). The VEGF assay was specific for the commonest VEGF isoforms, $VEGF_{165}$ and $VEGF_{121}$. The standard curve was prepared using recombinant human $VEGF_{165}$ provided with the assay.

Results

Sequence analysis

Genomic analysis revealed the absence of mutations in the coding region with four and one single nucleotide substitutions in the intronic and 5'UTR regions of the VEGF gene respectively (Fig. 1). One of these mutations was a G to A substitution in heterozygous state in the acceptor splice site of the intron 6 (IVS6-4).

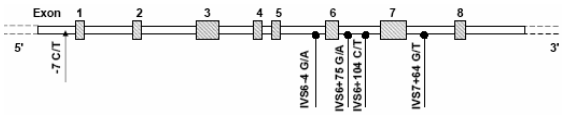

Fig. 1: Schematic representation of the VEGF gene. The mutation in the 5'UTR is designated by ▲ and the mutations in the intron region are denoted by ●

We have analyzed this variation using a website (http://www.itba.mi.cnr.it/webgene/) to verify the influence of this variation on the splicing process. This analysis revealed that such mutation did not change the accuracy of the mRNA splicing of the VEGF gene. The mutation in the 5'UTR at position -7 upstream to ATG start site, was a C to T transition. Using Matinspector online tools which identify transcription factor binding motif,[6] we analysed whether this sequence variation could change or create a new binding site for the transcription factors. A potential binding site for the *Sp-1* transcription factor was found in the 5'UTR

region from -6 to -15 bases. Therefore, the presence of the mutation at position -7 might affect the disruption of the *Sp-1* transcription factor binding site with subsequent reduction of VEGF gene transcription efficiency. To verify this hypothesis, we compared the VEGF plasma levels and the sequence variation –7 C/T in our patients. The presence of the T base change was not associated with VEGF plasma concentration (C=71.06 pg/ml, T=66.11 pg/ml). The heterozygous mutation was observed in 12 patients and the -7 C/T mutation in homozygous state was found in only one patient in which the corresponding VEGF plasma level was 49.5 pg/ml lower than non-carriers of the –7 C/T.

VEGF plasma levels

The mean level of plasma VEGF for patients with and without neovessels in the ischemic region was 68.86 and 66.64 pg/ml respectively. VEGF plasma levels showed no significant difference between patients with and without collateral blood vessels in ischemic myocardial area. The development of new vessels in the ischemic area was present in 62% of women compared to only 36% of male patients. The plasma levels of VEGF in women with collateral blood vessels or without new blood vessels were 55.41 and 73 pg/ml respectively without being statistically significant (Table 1).

Table 1: VGEF plasma levels of patients with collateral blood vessels (CBV)

Gender	CBV (%)	VEGF (pg/ml)
Female	62	55.4±19.4
Male	36	74.1±24.8

Discussion

In the present study, no significant changes were observed in plasma levels of VEGF for patients with and without neovessels in the ischemic area. Previous studies have reported both increasing serum VEGF levels in peripheral venous blood of patients with post-myocardial infarction as well as a much higher baseline levels of VEGF compared to healthy controls ; whereas 20-30 min post-procedure levels were reduced to healthy control values.[8,9] Another study suggested that plasma VEGF levels might not rise in the acute phase of MI during the first 24 hours, but were more likely to elevate over time, as they increased significantly in chronic MI patients of more than 3 months post-infarction.[10] This would reflect the observations of other studies with long-term follow-up. Interestingly, the results of our angiographic analysis for women and men revealed that females seemed to have a better chance of developing new capillary networks into ischemic myocardial area. It seemed that high VEGF plasma levels in MI female patients were not associated with the development of new collateral blood vessels.[11] In this study the elevated VEGF serum levels were significantly associated with worsened clinical outcomes and the increasing VEGF levels signified an early response to myocardial ischemia or infarction. According to these results, one may hypothesise that the increased VEGF serum levels, in patients with acute coronary syndromes, might show the increasing risk of myocardial cell necrosis. This may be secondary to thrombus formation at the culprit lesion and /or distal to embolization in coronary vascular beds.

Another study identified two sequences homologous to the consensus estrogens elements, of which one was located in the 5'UTR and the other in the 3'UTR of VEGF gene suggesting that the regulation of VEGF was probably mediated by transcriptional activation of the estrogens receptor.[12] Moreover, this study showed that estrogens elevated VEGF expression in the rodent uterus, human uterine tissue and HEC-1 cell-lines derived from human endometrial adenocarcinomas and human breast cancer cells. It would be interesting to investigate the functional significance of the estrogens response elements on regulation of VEGF transcription in both patients and healthy subjects. The molecular characterisation of the VEGF gene, performed in this study, showed that the sequence of this growth factor was highly conserved. The lack of genomic sequence variations might be in fact, due to the key role played by this growth factor during the vasculogenesis in the embryonic development.

As previously reported, loss of a single VEGF allele between days 11-12 of embryonic life was lethal in the mouse embryos.[13] Angiogenesis and blood-island formation were impaired, resulting in several developmental anomalies.[14] Therefore, we believe that the balance between angiogenic factors and angiogenesis inhibitors regulates angiogenesis. Numerous molecules have been reported to take part in angiogenesis. Among them, endothelium-specific factors and their signal transducing receptors have been studied extensively. Angiogenesis is a multistage multicellular process with well-organized interactions between numerous effector molecules and different cell types. Therefore variations observed in the angiogenesis process could be due to the cooperative roles played by the elements involved in the regulation of this delicate mechanism.

In conclusion, the molecular study of VEGF gene showed that the coding region of the VEGF growth factor gene is highly conserved. The lack of genomic sequence variations may be due to the key role which this growth factor plays during the vasculogenesis in the embryo development. The angiogenesis is a process highly checked by activator and inhibitor factors and therefore variations observed in this process could be due to those elements which play cooperatively for the regulation of this fine mechanism.

Acknowledgement

The authors would like to thank Shiraz University of Medical Sciences, Shiraz Iran and Fondazione Monzino and Fondazione CARIPLO of Italy for their financial supports.

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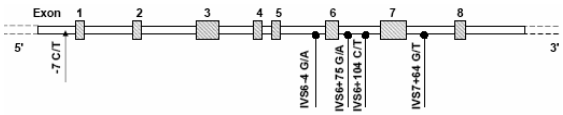

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